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(19) (CA) **CANADIAN PATENT** (12)

(54) Identification of Genes in Pseudomonas Bacteria

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ABSTRACT OF THE DISCLOSURE

Genetic analysis and modification of Pseudomonas bacteria is accomplished using a suicide plasmid which includes genes for replication in a narrow range of host cells and genes for conjugal transfer of the plasmid into a broad range of bacterial hosts, including Pseudomonas. The genetic donor is E coli and the plasmid carries a transposon having at least one antibiotic resistance marker, e.g. kanamycin resistance or tetracycline resistance, conferring thereon an antibiotic resistance not found in the Pseudomonas host or in the remainder of the suicide plasmid. The suicide vector is constructed and introduced into E coli cells, in which it replicates. Then it is introduced into Pseudomonas cells by conjugal transfer, whereupon the transposon inserts into the DNA of the recipient, deactivating or altering the activity of a gene therein, whilst the residue of the vector is eliminated and does not replicate. By this means, the operative symbiotic genes in the Pseudomonas may be identified, by comparison of the genetic behaviour of the modified Pseudomonas cells so formed with wild type Pseudomonas cells.

IDENTIFICATION OF GENES IN PSEUDOMONAS BACTERIA

FIELD OF THE INVENTION

This invention relates to transposon mutagenesis, and processes of mutagenesis of bacteria using vector plasmids. More particularly, it relates to vector plasmids for transposons such as Tn5 and Tn10, and their use in processes of mutagenesis and genome manipulation in Pseudomonas bacteria, and identification of genes therein.

BACKGROUND OF THE INVENTION

The Pseudomonas species of bacteria have a wide range of biological and industrial uses. For example, they are soil bacteria, which have interaction with plants. In some cases they adhere to or enter the root structure of the plant during cell division or replication. They appear to have a role in the preparation of plant growth hormone. They are also used in the industrial degradation of chemicals. Manipulation of the genes of Pseudomonas species offers the possibility of defining, controlling, and enhancing the various useful characteristics of Pseudomonas.

Before the genes involved in the industrial uses of Pseudomonas bacteria can be manipulated, enhanced or otherwise modified, they must be identified.

Chemical mutagenesis of bacteria (treating the bacteria with chemicals or radiation), to modify its DNA may generate

"multiple hit" damage in DNA rather than single point alterations, so that the resulting mutants have faults in more than one gene, and consequently such mutants may possess ambiguous characteristics.

Transposons are DNA sequences encoding antibiotic resistance and proteins necessary for transposition (transposases), which are capable of promoting introduction of their own DNA sequences to introduce additional or alternative genes into the genomes of bacteria. An example of a transposon which has been widely reported and used in genetic engineering experiments is Tn5, a bacterial transposon found in prokaryotic cells, for example E. coli. A review of the discovery, nature, properties and uses of the transposon Tn5 appears in "Biotechnology", July 1983, pp. 417-436 (Berg and Berg). Briefly, Tn5 is a discrete 5.7 kilobase (kb) segment of bacterial DNA which can insert at high frequency into numerous sites in the chromosomes, plasmids and temperate phages of Gram negative bacteria. It comprises a pair of end sequences, each of 1.5kb length and virtual repeats of each other, and a central segment whose sequence is unrelated to that in the repeats but which contains a kanamycin-resistance gene.

Transposon mutagenesis is specific and useful since one knows and can identify by restriction mapping the DNA sequence of the transposon being inserted into the host bacteria. Transposable elements often encode phenotypes such as antibiotic markers by which presence they can be recognised. The inserted transposon has the effect of inactivating or otherwise modifying

the activity of the gene of the bacterial DNA into which it inserts.

In order to incorporate the desired transposon DNA into the bacterial DNA of Pseudomonas, one should ensure that the transposon DNA portion is incorporated therein by transposition but that the remaining DNA of the plasmid is not. What is required therefore is a plasmid carrying a suitable transposon which can transfer itself conjugatively to Pseudomonas cells so that its transposon portion can transpose into the bacterial DNA, but such that the remainder of the plasmid vector will be eliminated from the bacterial cell - a "suicide plasmid".

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel process for effecting mutagenesis in Pseudomonas, using suicide plasmids incorporating a transposon such as Tn5 or Tn10.

It is a further object of the invention to provide novel, genetically modified Pseudomonas bacteria.

The present invention provides a process for identifying and modifying genes of the bacteria Pseudomonas which involves the use of vector plasmids incorporating a transposon having an antibiotic resistance marker to which antibiotic wild type Pseudomonas are sensitive, for example kanamycin and tetracycline. Examples of suitable such transposons are Tn5, which encodes Kan^r and Tn10, which encodes Tc^r. The vector plasmids are suicidal in Pseudomonas and therefore suitable for mutagenesis with these transposons. The vectors used in the present invention are composed of a suitable replicon which

functions in E. coli but not in Pseudomonas and a region encoding the N-type or W-type of bacterial conjugation system (N-Tra genes or W-Tra genes) that function in matings between E. coli and Pseudomonas. They are plasmids which have a wide host range of efficient conjugal transmission, but with a capacity of replication and maintenance limited to E. coli and closely related bacteria. Accordingly, they can be produced and maintained in E. coli cells, the E. coli cells containing them can then be mated with Pseudomonas cells, but the plasmid is suicidal upon entry into the Pseudomonas cells. Accordingly, the transposon portion can enter the DNA of the receiving Pseudomonas cell, but the plasmid portion fails to replicate therein. The chosen transposon has an antibiotic resistance different from or additional to that of the host Pseudomonas cells. Thus one avoids use of transposon Tn1, for example, which confers ampicillin resistance, a feature already possessed by the wild-type Pseudomonas. One can select from the progeny of Pseudomonas cells the derivatives carrying transposon-insertions, based upon the specific antibiotic resistance conferred thereon by the transposon, and from among these, by comparison with the behaviour of wild type Pseudomonas cells, locate those in which various useful, characteristic properties have been modified.

The present invention thus also provides a process for producing genetically modified Pseudomonas bacteria which contain transposon sequences derived from the aforementioned transposon-containing plasmid vectors. In the process, the

appropriate plasmid vector is replicated in E. coli bacterial cells, the E. coli cells containing the plasmid vectors are conjugated with the recipient Pseudomonas cells, and from the progeny Pseudomonas there are recovered those containing transposon sequences derived from the plasmid vector. Conjugation is a much more efficient procedure than transformation, for producing strains carrying transposon-insertions from a suicide vehicle. Accordingly the suicide vehicles of the present invention are conjugative.

In the accompanying drawings:-

Figure 1 is a diagrammatic representation of novel plasmid pMKK10 and the steps in its preparation;

Figure 2 is a diagrammatic representation of novel plasmid pMKK23 and the steps in its preparation;

Figure 3 is a diagrammatic representation of a process for preparing novel suicide vector plasmid pMKK10::Tn5 according to the invention;

Figure 4 is a diagrammatic representation of a process for preparing novel plasmid pMKK11 and novel suicide vector plasmid pMKK11::Tn10 according to the invention.

Figure 5 is a diagrammatic representation of radiation sensitive film images obtained from DNA of Pseudomonas cells hybridized with ^{32}P labelled λ::Tn5 probe, in accordance with Example 3 below.

PREPARATION OF THE SUICIDE VECTOR PLASMID

The vector plasmids used in the present invention have firstly the ability to replicate in E. coli but no ability to

replicate in Pseudomonas. Thus they contain an origin of replication derived from an E. coli plasmid which has a narrow range in terms of acceptable host bacterial species. An example of a suitable such E. coli plasmid is plasmid pBR329. However, the vector plasmids do not contain any replication genes which confer the ability to replicate in a wide range of bacterial species. Secondly, they have the ability to conjugate with relatively high efficiency between E. coli and Pseudomonas bacterial species, i.e. they contain appropriate N-type or W-type transfer genes, and thus are conjugal transfer proficient. Thirdly, they contain at least one antibiotic resistance marker, on the transposon, which incorporates into the Pseudomonas. The antibiotic resistance is different from that naturally possessed by the Pseudomonas, to allow for the separation and selective cultivation and growth of those cells which have accepted the transposon.

Figure 1 of the accompanying drawings illustrates diagrammatically the steps in a process of preparing one specific, exemplary suicide vector plasmid for use in the present invention. The known E. coli plasmid pCU1, characterized in detail by the inventor and serving as a prototype of Inc.N group of plasmids (see Konarska-Kozlowski and Iyer, 1981, Can.J.Microbiol., 27 p616-626), is, in this example, used as the starting plasmid. It contains N-type transfer genes, various antibiotic resistance genes, and a Pst I restriction site disposed adjacent one end of the N-type tra genes. The initial objective is to excise the tra gene for ligation into the suicide plasmid. The transposon element Tn5

also has a Pst I restriction site, located near one end of one of its repeat segments, and remote from its Kan^r gene. Accordingly, E. coli cells containing plasmid pCU1 are infected with phage λ::Tn5, and then selected for kanamycin resistance, to obtain those containing plasmids which have successfully obtained Tn5 insertions. The Tn5 inserts randomly into the plasmid DNA. Next, it is necessary to isolate those which have the Tn5 element inserted at the desired location, i.e. close to but beyond the end of the tra region. Those which have Tn5 inserted within the tra sequences will in consequence have had their transfer genes inactivated. They can be eliminated by mass mating of colonies of the Tn5 containing E. coli cells with another Kan^s E. coli strain, but one which exhibits an additional phenotype not found in the Tn5-containing E. coli strain e.g. rifampicin resistance and selecting from among the progeny those exhibiting kanamycin resistance and the additional phenotype (rif^r), to isolate those containing the plasmids having Tn5 incorporated outside the tra region. Colonies of these cells are grown, their DNA is extracted and analyzed by cleaving the DNA with appropriate restriction endonucleases, and those having the Tn5 sequence in the desired location, i.e. those labelled pCU1::Tn5 in Fig. 1 thus determined. From these, fragments consisting essentially of the tra region can be obtained by use of Pst I endonuclease. The Pst I fragment containing the transfer region is next ligated with Pst I cut pBR 329, a known E. coli plasmid, to form novel suicide vector plasmid pMKK10, by standard techniques.

It will thus be appreciated that an essential function

of the transposon Tn5 inclusion in modified plasmid pCU1::Tn5 is to provide an additional Pst I restriction site at the correct location on the plasmid. It allows the production of a fragment containing the Tra region but omitting the Rep gene, by use of the same restriction enzyme (Pst I) as can be used to cut pBR329, with which it is to be ligated to form suicide vector pMKK10. Other means could be adopted to introduce an appropriate restriction site at the appropriate location, such as introduction of linkers (relatively small natural or synthetic DNA sequences) bearing appropriate sequences, by known techniques. However, the use of transposons for this purpose is preferred, on account of its convenience. The Tn5 also provides an antibiotic resistance marker, for ease of selection during the process.

Fig. 2 of the accompanying drawings similarly diagrammatically illustrates the preparation of an alternative novel plasmid pMKK23, which contains W-transfer gene instead of N-transfer genes. The starting plasmid is S-a322, which is a derivative of known and commercially available plasmid S-a. It similarly possesses a Pst I site adjacent the end of the W-tra gene sequence, and is similarly provided with a transposon Tn5 sequence adjacent the other end of the W-tra gene sequence so that a portion consisting essentially of the tra gene can be excised therefrom with Pst I and ligated with pBR329, to prepare novel plasmid pMKK23. This plasmid can be similarly infected with λ::Tn5 or λ::Tn10 to produce suicide vectors effective in transconjugants into Pseudomonas host cells to insert Tn5 and Tn10 sequences thereinto.

It will be noted that plasmid pBR329, in common with many E. coli plasmids suitable for constructing suicide vectors for use in introduction of transposons into host cells, contains an ampicillin resistance gene Ap^r. Its Ap^r gene contains a Pst I site, so that on cleavage with Pst I to form pMKK10 or pMKK23, the ampicillin resistance is inactivated, and the resulting novel vector plasmids are tetracycline resistant, as indicated in Figs. 1 and 2, so as to provide a selectable marker.

Next, as further illustrated in Fig. 3, entire transposon Tn5 is re-introduced into pMKK10 (or similarly into pMKK23). This is accomplished by introducing pMKK10 into appropriate host cells, preferably E. coli cells, by conjugation or transformation. The cells are then infected with a bacteriophage containing the transposon Tn5, e.g. λ b221 c1857 Pam Oam rex :: Tn5 (hereinafter λ:: Tn5), which is generally available and described in the prior art literature. By a process of genetic transposition in the host cell, transposon Tn5 is inserted into pMKK10, to produce suicide vector plasmid pMKK10::Tn5, as shown in Fig.3. The genetic transposition is conducted under special conditions so that the phage will not replicate. Plasmid pMKK10::Tn5 will replicate in host E. coli cells. Those cells which contain pMKK10::Tn5 resulting from successful transposition can be isolated by selective cultivation on account of their kanamycin resistance conferred by transposon Tn5.

The E. coli cells containing plasmid pMKK10::Tn5 are

then mated en masse with cells of Pseudomonas. The presence of the Tra region ensures efficient conjugation, whilst the plasmid pMKK10:Tn5 does not replicate in the Pseudomonas. As a result, transposon Tn5 leaves the plasmid to insert elsewhere in the DNA of the recipient Pseudomonas whilst the vector residue is eliminated.

The presence of a Tn5 transposon imparts to the cell antibiotic resistance according to the antibiotic marker on the Tn5 itself, e.g. kanamycin resistance. Cells which have successfully accepted Tn5 can therefore be separated and selected from those which have not, by cultivation in a medium containing the appropriate antibiotic namely kanamycin. Transposon Tn5 also has regions coding for streptomycin resistance. Cultivation in a medium containing streptomycin, however, does not select only the cells which have accepted the transposon, since Pseudomonas has natural streptomycin resistance, to a degree depending upon the specific strain.

The non-transposon region of pMKK10 also has a sequence coding for tetracycline resistance. Use can be made of this, to determine the presence in the Pseudomonas cells of residual pMKK10 viable plasmid. The cells found to be resistant to kanamycin and also found to be resistant to tetracycline have incorporated therein the plasmid pMKK10 in viable form, i.e. the plasmid was not suicidal. In practice, it is found that this does not happen to any significant extent.

Fig. 4 of the accompanying drawings is a similar diagrammatic illustration of the preparation of a specific,

exemplary suicide vector plasmid for delivering transposon Tn10 to Pseudomonas cells. In this instance the starting plasmid is pMKK3 (which is based on known, available plasmid pCU101, Thatte and Iyer, 1983, Gene 21, 227-236) to which the Tn5 element has been transposed. The Tn5 sequence contains a Bam HI restriction site which is distant from the kanamycin resistance coding sequence. The plasmid pMKK3 has a second Bam HI restriction site, which is distant from the transposon Tn5 and from the N-transfer genes. The region between the two Bam HI sites does not include the Km^r gene. Accordingly, the pMKK3 is treated with Bam HI endonuclease, and religated. Deletion of small Bam HI fragment generates a plasmid designated as pMKK11. This plasmid pMKK11 confers upon cells resistance to Km and Cm. It contains just one half of the Tn5 element encoding Km^r. The transposable function of Tn5 has been eliminated. The pMKK11 is then treated, in E. coli cells, with phage λ::Tn10 in a similar manner to that previously described in constructing pMKK10::Tn5, the plasmid molecules which have successfully incorporated Tn10 at a location outside the N-transfer region are selected as before, to produce novel suicide vector plasmid pMKK11::Tn10 as shown. The tetracycline resistance Tc^r of the transposon is retained.

The Tn5 and Tn10 transposon insertion into the

Pseudomonas DNA should be essentially random in nature, into a chromosome or a plasmid thereof. Since each Pseudomonas cell will accept one transposon, each such cell will as a result have a single genetic modification, normally a single gene inactivation as compared with the wild type of the species. The various cells can now be separated according to their modified phenotype.

Further, they can be analysed to identify the gene which has been deactivated, preliminary to its replacement, enhancement or other genetic manipulation in vivo or in vitro.

This analysis is done, in respect of mutant Pseudomonas cells containing transposon Tn5, by removing the DNA from the cell in the known way, and then using restriction enzyme EcoRI which cuts out the portion of the DNA chain containing the transposon Tn5 insert, along with a DNA sequence on either side of the site of Tn5 insertion. Effectively therefore EcoRI cuts out as a DNA fragment the gene, or at least a portion of the gene, which has been deactivated by the insertion therein of Tn5. Since the DNA sequence of Tn5 is well known, sequence analysis of the excised fragment by standard, known methods will allow dissection of the structure of the gene.

The excised piece of DNA may also be radioactively labelled, and used as a probe against DNA fragments obtained from a wild type Pseudomonas cell. Using a Southern-blot type hybridization (Southern, E.M., 1975 J. Mol. Biol. 98, 503-517), the DNA fragment to which the radioactively labelled DNA containing the transposon hybridizes, through DNA-DNA homology, can be identified. This DNA sequence so identified is at least

a portion of the gene responsible for the phenotype of the cell which the transposon altered upon insertion. The DNA so identified can therefore be isolated from the gel, introduced into a plasmid by enzymatic ligation, replicated in a cell, sequenced and manipulated etc., to vary or enhance the phenotype characteristic of the Pseudomonas cell.

The processes of incorporating transposons into Pseudomonas by means of suicide vectors according to the invention, as described herein, thus give valuable analytical information and methods of testing concerning genetic structure and gene information in the Pseudomonas bacterial species. By isolation of various mutants containing transposons as described herein, and comparisons thereof with wild type Pseudomonas bacteria, one can determine which functional gene has been deactivated by the presence of the transposon. By analysis of the DNA region into which the transposon has been inserted, the nature and structure of a specific gene can be determined. Novel and industrially advantageous features of Pseudomonas can be defined.

The invention is further described and illustrated in

the following specific, non-limiting examples. In the specific experimental examples, the following general techniques and materials were employed.

Bacterial Strains

The bacterial strains used in the experiments are listed, along with some of their characteristics, in Table 1 below:

TABLE 1

Strain	Relevant Characteristics
E. coli	
RR1	Pro^-, leu^-, thy^-, thi^-, Str^-
HB 101^{rif}	as RR1 except $recA^-$, rif^r
Pseudomonas	
P. putida P008	Met^-, nal^r, rif^r
P. aeruginosa P009	Leu^-, nal^r, Tc^r, rif^r
P. stutzeri	
F.42	Wild type isolate

Abbreviations:

leu - leucine; Met - methionine;
nal - nalidixic acid; r - resistance;
Rif - rifampicin; Str - Streptomycin;
thi - thiamine; thy - thymine;
rec A^- - deficient in general recombination

Plasmids

The plasmids employed as described in the accompanying drawings and in Table 2 below.

TABLE 2

Plasmid	Relevant Characteristics	Derivation (if not in public literature
pCU1	Tra^+(N-type), N-group replicon, Ap^r; Sm^r/Sp^r	
pCU1::Tn5	as pCU1, plus Km^r	Tn5-carrying derivative of pCU1
pBR 329	pMB1 replicon, Ap^r, Tc^r	
pMKK10	Tra^+ (N-type), pBR329-(pMB1-replicon), Tc^r	
pMKK10::Tn5	as pMKK10, plus Km^r	Tn5-carrying derivative of pMKK10
pSa 322	Tra^+(W-type), pMB1 replicon, Ap^r	A. Kado,
pSa 322::Tn5	as pSa 322 plus Km^r	Tn5-carrying derivative of pSa322
pMKK23	Tra^+ (W-type), pBR329 (pMB1-replicon), Tc^r	
pMKK23::Tn5	as pMKK23 plus Km^r	Tn5-carrying derivative of pMKK23
pMKK11	Tra^+ (N-type), pACYC 184 (p15A-replicon, Km^r, Tc^s	Bam HI treatment of pMKK3
pMKK11::Tn10	Tra^+ (N-type), pACYC 184 (p15A-replicon), Km^r, Tc^r	Tn10-carrying derivative of pMKK11

Abbreviation:

Ap - ampicillin; Km - kanamycin;

Sm - streptomycin; Sp - spectinomycin; Tc - tetracycline;

Tra - conjugal transfer proficiency.

Bacteriophages and Transposons

Bacteriophage b221 c1857 Pam Oam rex :: Tn5, referred to herein as λ:: Tn5 is described in the literature -- see W. G. Shanabruch and G. C. Walker, "Localization of the plasmid (pKM101) gene(s) involved in rec A^+ lex A^+ - dependent mutagenesis", Mol. Gen. Genet. 179: 289-297 (1980).

λ_{561} = λb221 CI857 CI::Tn10 O_{29} P_{80}, gift from N. Kleckner.

Media, chemicals and biochemicals. For the growth of E. coli strains, Tryptone-Yeast extract-sodium chloride (TYS) medium was used at 37°C in TY medium containing $CaCl_2$. For the growth of Pseudomonas strains, Pseudomonas fluorescent agar (PAF) was used. Plasmid-carrying strains were grown in medium containing an antibiotic to which the plasmid specified resistance. For solidifying media, Difco agar was used at 1.5% (w/v). The concentration of antibiotics used in solid media was as follows (μg/ml): Kanamycin, Km 50; spectinomycin, Sp, 50; streptomycin, Sm, 50; tetracycline, 20 rifampicin, Rif, 150. In liquid media, half of these antibiotic concentrations were used.

Bacterial matings and transformation. Spot-matings between 10^6 donors and recipients were done on appropriate selective plates. Alternatively, 10^8 cells of donors and recipients were mixed on membrane filters and the filter was incubated on PAF agar

plates for 4-12h, followed by plating on appropriate selective plates.

Genetic transformation of E. coli was carried out by the method of Cohen. et al Proc. Natl. Acad. Sci. U.S.A. 69, 2110-2114 (1972), with the following change. The cells were washed with 0.03 M $CaCl_2$ instead of 0.01 M NaCl. This prevented the clumping of cells.

Plasmid DNA manipulations. The plasmid DNA preparations used in all cloning and transformation experiments were isolated and purified by the procedure described in Konarska-Kozlowska, and Iyer, "Gene", 14 (1981), p.195-204.

Plasmid DNA used in restriction mapping was isolated by the rapid method of Brinboim and Doly 1979, Nucleic Acids Res. 7, 1513-1523, or Holmes and Quigley, Anal. Biochem. 114: 193-197, (1981). Restriction enzymes were used as recommended by the supplier. Gel electrophoresis was carried out in 0.9% agarose gels in Tris-acetate buffer (0.4M Tris, 0.2M sodium acetate, 0.1M EDTA, pH 8.0 adjusted with acetic acid) at 2 volts/cm for 12 hrs. HindIII fragments of bacteriophage DNA were used as size standards. DNA ligation reactions were performed at 14°C for 12-20 hrs. with T4 DNA ligase in a buffer recommended by the supplier.

Colony-hybridization. ^{32}P-labelled DNA of pMKK10 or pMKK23 :: λTn5 were prepared by nick-translation (see Rigby et al, J. Mol. Biol. 113: pp. 237-251, 1977) and were used as probes in colony-hybridization. The nitrocellulose sheets (Schleicher and Schuell, BA85 or NEN New England Nuclear Colony-Screen Filters) carrying the colony blots were hybridized according to the procedure suggested by the supplier. Strains of E. coli carrying pMKK10, pMKK10::Tn5 or pMKK23 were used as controls.

Example 1

Suicide vehicles were developed for conjugative insertion into Pseudomonas strains.

E. coli strain HB101^{rif}, i.e. E. coli strain HB101 as listed in Table 1 but containing recombinant plasmid pMKK10 (Fig. 1) prepared by cloning a region of the N-group plasmid pCU1 specifying conjugal transfer (N-type tra genes) into plasmid pBR329 and not containing any transposon-like structures, was infected with the bacteriophage λ::Tn5 (Fig.3), and the resulting kanamycin-resistant colonies were mated en masse with rif^r E. coli strain HB 101^{rif}. The Km-resistant transconjugants of HB 101^{rif} were further tested for conjugal transmission of Km^r and tetracycline-resistance (Tc^r, pMKK10-associated marker). Plasmid DNA from one such derivative that transferred both Km^r and Tc^r at a frequency of 1 (per donor cell) was analysed by restriction analysis for the presence and location of the transposon. This plasmid was designated as pMKK10::Tn5, and its simplified gene map, along with its schematic preparation, are shown in Fig. 3.

By an essentially similar procedure, a plasmid designated pMKK23::Tn5 was prepared, from pMKK23 illustrated in Fig. 2, and containing W-type transfer genes.

Example 2 - Suicide Plasmid Carrying Tn10

By similar processes as described above and illustrated in Fig. 4, a suicide plasmid designated pMKK11::Tn10 containing transposon Tn10 was prepared. This plasmid was constructed, selected and isolated by the standard techniques described

above, by first creating pMKK3::Tn5 from pCU101 and phage λ::Tn5, cutting and ligating pMKK3::Tn5 with Bam HI endonuclease to incorporate Km^r and to delete sequences responsible for transposition, so that it cannot move from the plasmid to other genetic elements, followed by infection of the E. coli cells containing plasmid pMKK11 with phage λ::Tn10 in the manner described above, and selection of transconjugants containing Tn10, on the basis of Tc^R. Vector pMKK11::Tn10 has tetracycline resistance Tc^r encoded on the transposon and kanamycin resistance Km^r encoded on the plasmid allowing for double selection. With Tc^r encoded on the transposon rather than the plasmid, distinction from cells containing intact plasmid, on the basis of Tc^r is provided.

Transposon Tn10 is a well-characterized transposon that specifies resistance to tetracycline (Tc^r).

Example 3 - Transposon mutagenesis in Pseudomonas and other Gram-negative bacteria

Various strains of Pseudomonas were mutagenized using the suicide plasmid vector pMKK10::Tn5. The reagents and procedures were generally as previously described. Table 3 below lists the bacterial strains employed, and the qualitative conjugal transfer ability observed.

In the experimental procedures, donors and rcipients were mid-log phase cultures grown in L broth. For Pseudomonas recipients, mixtures of donor and recipient strains (5 ml + 5 ml) were mated on filters for 4-5h, resuspended and plated on 20 Kan rif agar (Kanamycin 150 ug/ml,

Rifampicin 150 µg/ml). The count of donor cells was simultaneously made on rif agar, and the transconjugant frequency was scored per donor cell. Transconjugants carrying Tn5 were identified by colony hybridization using a λ::Tn5 probe, with ^{32}P labelling. The colonies are lysed on nitrocellulose film, to release therefrom and bind the cellular DNA. Those DNAs containing the Tn5 sequence hybridize with the radioactive probe, to produce dark spots on the recording film (Figure 5).

TABLE 3

CONJUGAL TRANSFER OF pMKK10::Tn5

Recipient Strain	Transfer Ability	
Pseudomonas:		
PO08	++	(fair)
PO09	+++	(good)
F42	++	(fair)
stutzeri	+++	(good)
Escherichia (control)	++++	(very good)

The results of bacterial matings (genetic data) involving the Pseudomonas strains is given below in Table 4. In each case the donor strain was E. coli HB101 rif. The measure of transfer frequency is effectively a measure of both the incorporation of the suicide plasmid vector pMMK10::Tn5 into the recipient Pseudomonas strain, and the survival of the suicide plasmid vector therein as a whole, discrete plasmid. The transconjugants were selected on PAF plates supplemented with 150 ug kanamycin and 150 ug rifampycin.

TABLE 4

Conjugal Transfer of PMKK10::Tn5 (quantitative)

Recipient Strain	Transfer Frequency Km	Co-inheritance of unselected markers* Tc (%)
Pseudomonas		
POO8	1×10^{-6}	3
F42	4×10^{-5}	3
stutzeri	4×10^{-5}	4
E. coli^{rif}		
HB101	1	100

*The transconjugants were selected on kanamycin agar and then screened for co-inheritance of tetracycline resistance.

Molecular analysis of the recipient cell plasmids showed random Tn5 transposition. This was performed by preparation of total DNA from kn^r transconjugants and digestion of the DNAs with EcoRI restriction enzyme. The digested DNA is Southern-blotted and hybridized to appropriate probes. The hybridizations revealed that in the majority of cases there was a true transposition at different sites of the Pseudomonas genome. In a minority of cases there was true transposition accompanied by co-integrate formation but this has been resolved by growing transconjugants on non-selective media.

As shown in Fig. 5, DNA from transconjugants of P. stutzeri, P p008 and P F42 exhibit strong hybridization to radioactive probe λ::Tn5 showing that the transconjugants are due to Tn5 insertions. Similar hybridization occurred to the DNA of pMKK10::Tn5, demonstrating a positive control. No hybridization was

evidenced with DNA from P. stutzeri, P. PA08 or P F42 which had not been transconjugated, thereby providing a negative control.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process of preparing genetically modified mutants of Pseudomonas bacteria, which comprises conjugating viable cells of said Pseudomonas with viable E. coli cells which contain a suicide plasmid vector, said vector including:

an origin of replication derived from an E. coli plasmid;

Tra genes derived from an E. coli plasmid;

and a transposon bearing at least one antibiotic-resistance marker sequence which is different from any antibiotic resistance of said Pseudomonas;

said vector being essentially free of rep genes having a broad range of acceptable host cells.

2. The process of claim 1 wherein said transposon is Tn5 or Tn10.

3. The process of claim 2 wherein the vector contains N-type transfer gene or W-type transfer gene.

4. The process of claim 1 wherein the suicide vector plasmid is selected from pMKK10::Tn5, pMKK23::Tn5, and pMKK11::Tn10.

5. The process of claim 1 which includes the subsequent step of isolating the Pseudomonas mutants so formed by selective cultivation of the conjugation products in a medium containing said antibiotic.

6. A process of producing genetically modified mutants of Pseudomonas bacteria which contain transposon sequences, which comprises:

replicating in E. coli cells a plasmid vector which is suicidal in Pseudomonas, contains an origin of replication derived from an E. coli plasmid, contains Tra genes derived from an E. coli plasmid, contains a transposon bearing an antibiotic-resistance marker sequence conferring thereon resistance to an antibiotic to which the Pseudomonas is not naturallly resistant, and is essentially free of rep genes having a broad range of acceptable host cells;

conjugating E. coli cells containing said suicide vector with Pseudomonas cells, to transfer said plasmid vector thereto;

and isolating from the modified Pseudomonas cells so treated those which have incorporated therein the transposon, by cultivation of the cells so treated in a cultivation medium containing appropriate amounts of said antibiotic for which the transposon encodes resistance.

7. A process of identifying and analysing genes in Pseudomonas bacteria, which comprises

preparing genetically modified mutants of Pseudomonas by the process of claim 1;

isolating a specific mutant from the products of said process on the basis of predetermined modified phenotype in comparison with natural Pseudomonas bacterial cells,

treating the DNA of said mutant with an appropriate

restriction enzyme to separate out a DNA sequence therefrom which contains the transposon sequence;

and analysing the DNA sequence thus separated out.

8. The process of claim 7 wherein the genetically modified mutant contains in its DNA a transposon sequence selected from transposon Tn5 and transposon Tn10.

9. The process of claim 7 wherein the genetically modified mutant contains in its DNA transposon Tn5.

10. A vector plasmid suitable for introduction of transposon into Pseudomonas host cells, said vector plasmid containing: an origin of replication which is operative in an E. coli host cell but inoperative in a Pseudomonas host cell; appropriate N-type or W-type transfer genes for conjugal transfer proficiency between E. coli cells and Pseudomonas cells; and a transposon containing an antibiotic resistance marker, the antibiotic resistance thus conferred being different from antibiotic resistance naturally possessed by the Pseudomonas.

11. A vector plasmid according to claim 10 containing N-type transfer genes.

12. A vector plasmid according to claim 11 which is pMKK10::Tn5.

13. A vector plasmid according to claim 11 which is pMKK11::Tn10.

14. A vector plasmid according to claim 10 containing W-type transfer genes.

15. A vector plasmid according to claim 14 which is pMKK23 carrying a Tn5 transposon, namely pMKK23::Tn5.

16. A vector plasmid according to claim 14 which is pMKK23 carrying a Tn10 transposon, namely pMKK23::Tn10.

17. Plasmid pMKK10.

18. Plasmid PMKK11.

19. Plasmid pMKK23.

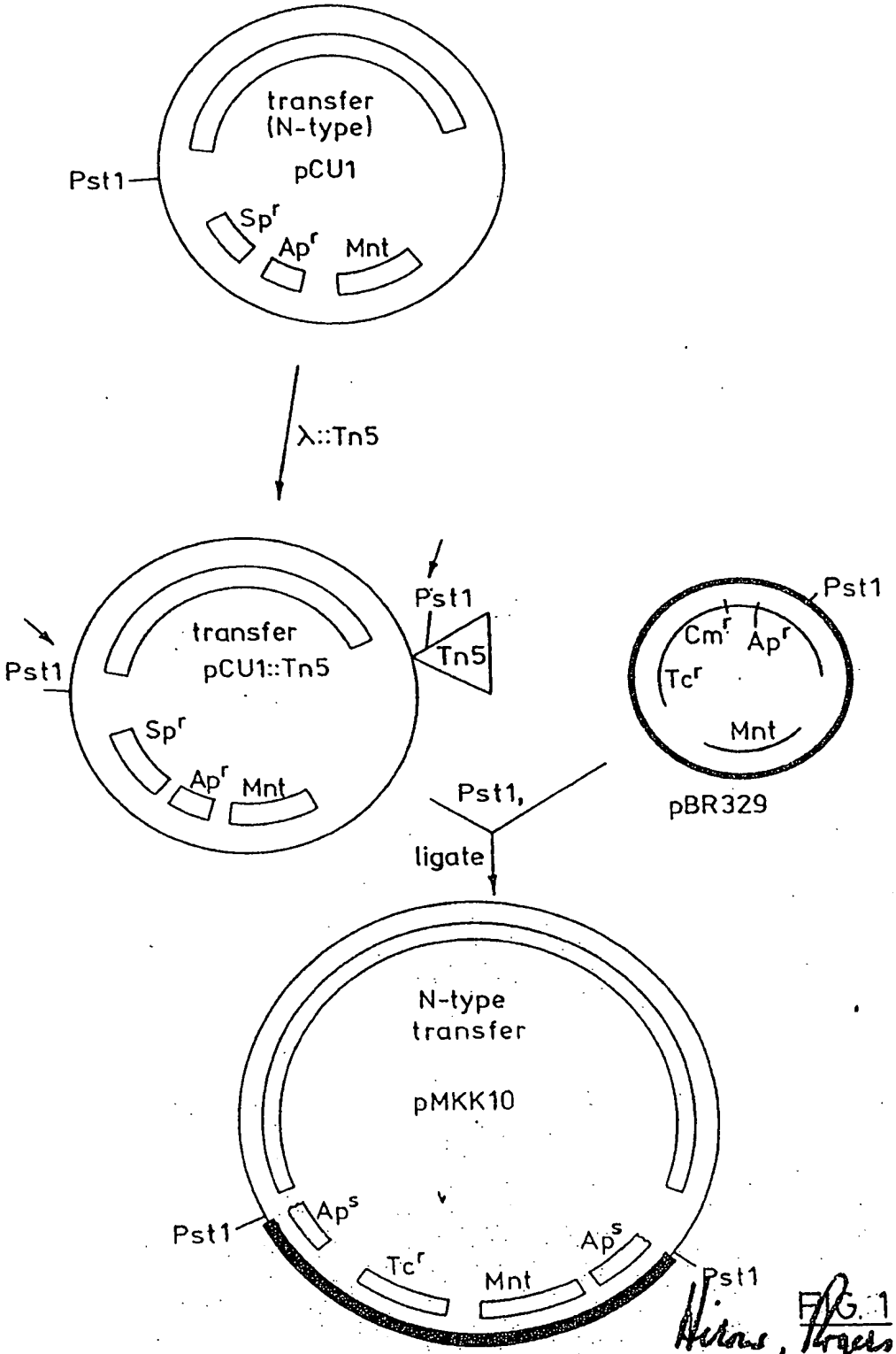

FIG. 1

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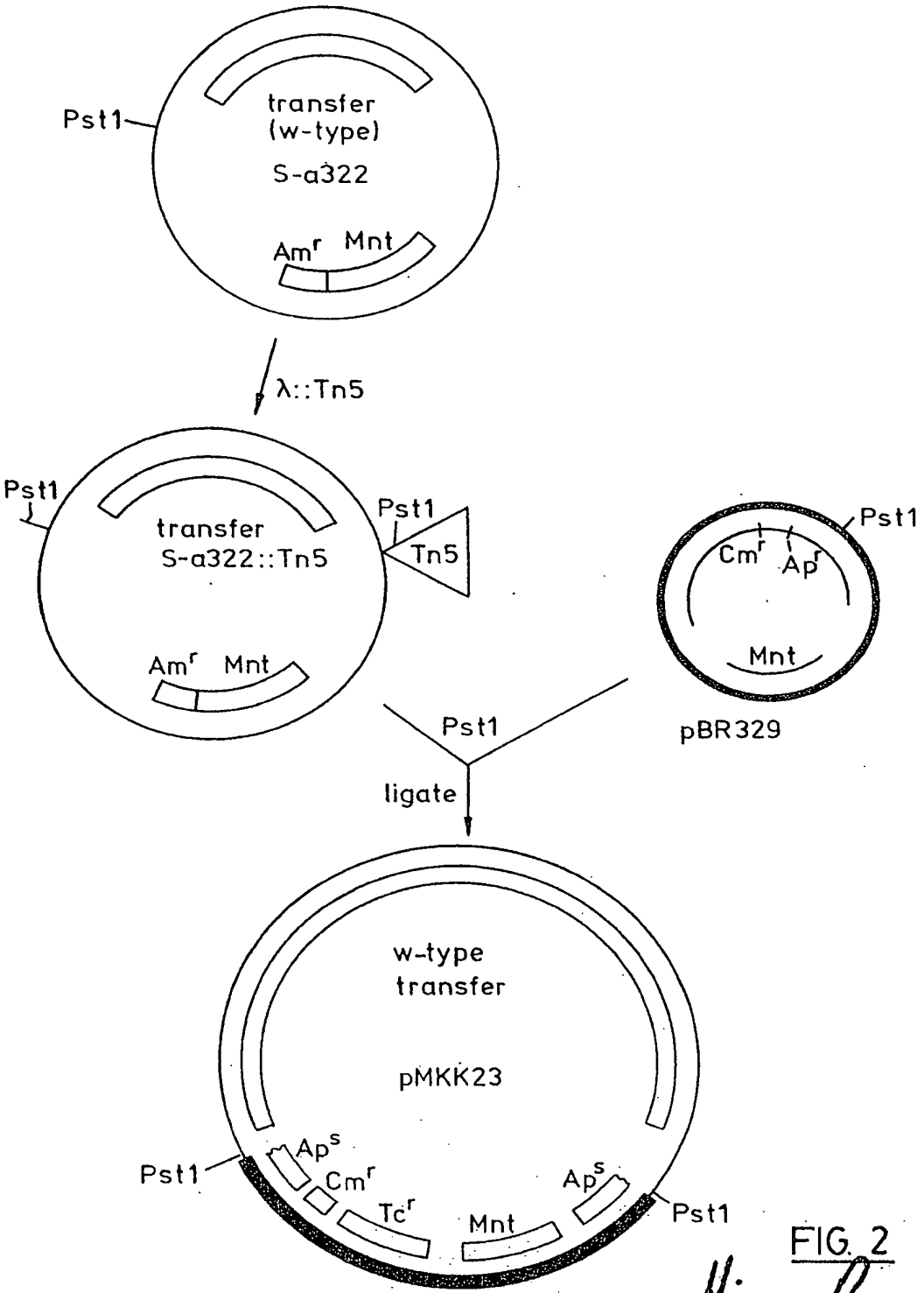

FIG. 2

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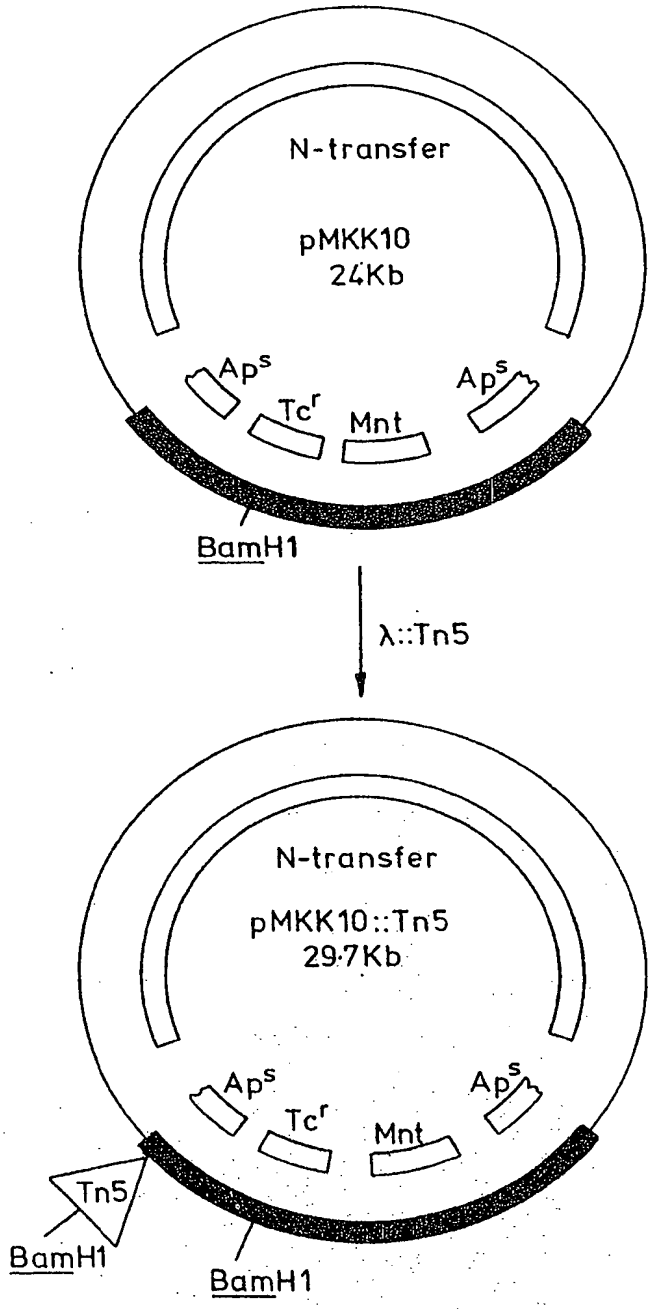

VEHICLE DELIVERING Tn5 ELEMENT

FIG. 3

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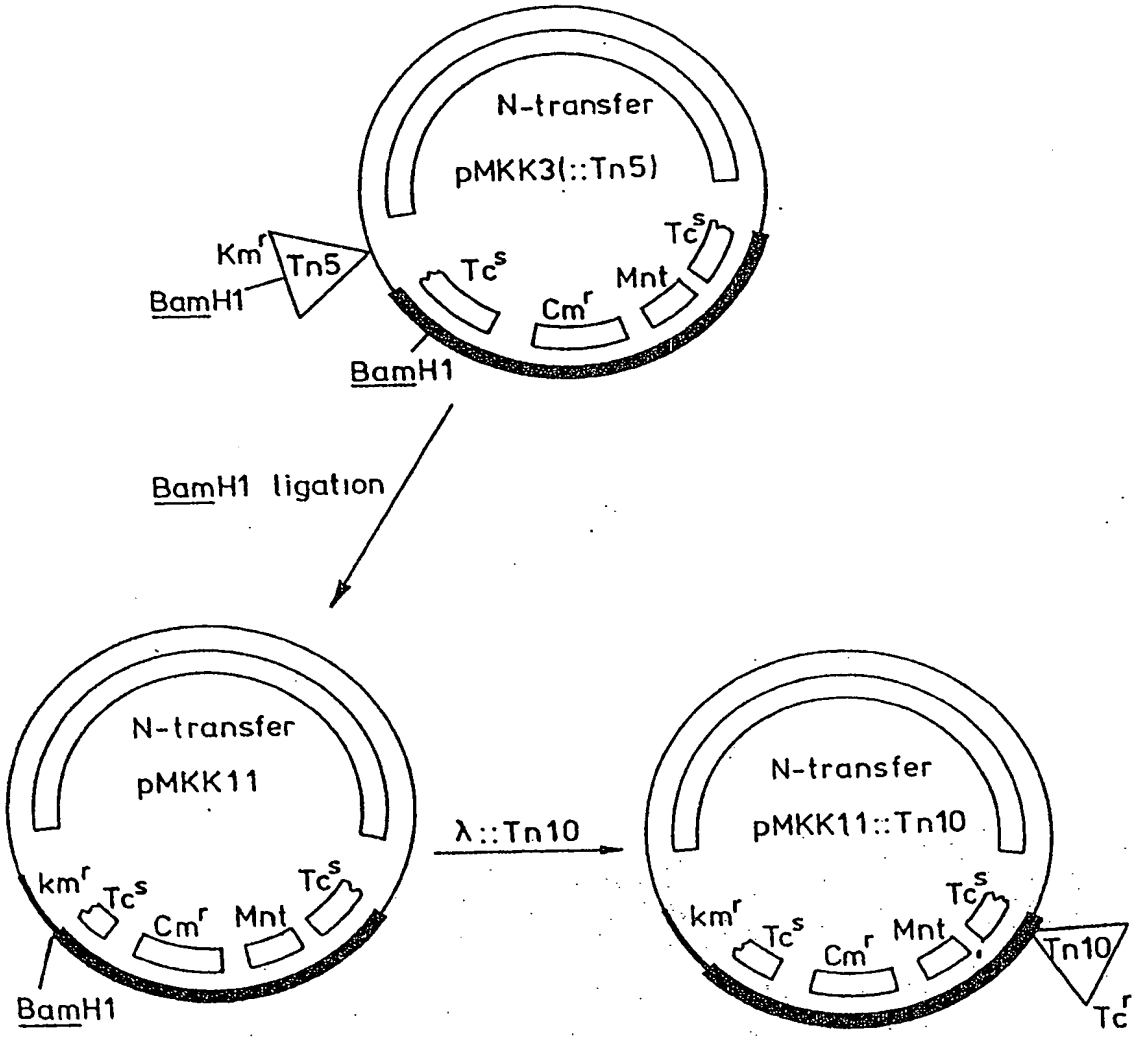

VEHICLE DELIVERING Tn10 ELEMENT

FIG. 4

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Probe: λ::Tn5 (^{32}p)

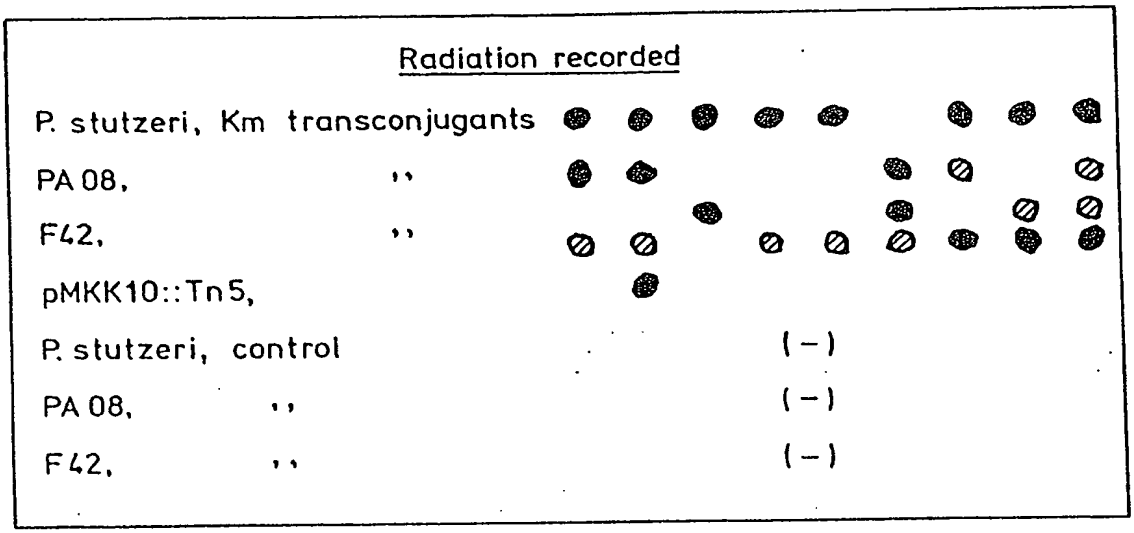

FIG. 5

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